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Bactericidal and Fungicidal Activity of *Aspergillus ochraceus* Metabolites and Some Derivatives

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Abstract: The bactericidal and fungicidal activities of aspyrone and asperlactone, secondary metabolites of *Aspergillus ochraceus*, and some aspyrone derivatives were studied. In general, aspyrone exhibited better activity than asperlactone or aspyrone derivatives. The inhibition patterns of the assayed compounds were different. *Helminthosporium monoceras* was the tested mould most inhibited by the studied compounds. The comparative study of the activity of the different compounds showed that the epoxy group seems to be necessary for activity against some micro-organisms. © 1998 SCI

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Key words: aspyrone; asperlactone; aspyrone derivatives; bactericidal and fungicidal activity; secondary metabolites; *Aspergillus ochraceus*

1 INTRODUCTION

Intensive screening of micro-organisms for substances of value in medicine or agriculture has revealed the wide range of biological activities possessed by secondary metabolites. Before the antibiotic era, relatively few microbial products were discovered as a result of their biological activity. One exception was the group of fungal metabolites called ergot alkaloids; a persistent effort by chemists to purify the pharmacologically active principle of ergot culminated in the isolation of ergotine[1] in 1907. The biochemical basis of microbial antagonism was determined during the late nineteenth century, but the nature of the agents responsible did not attract strong interest amongst chemists. The antibacterial fungal metabolite first described by Gosio[2] in 1896 was probably mycophenolic acid, reisolated in 1913 as a crystalline metabolite of *Penicillium stoloniferum* Thom., and subsequently discovered in various fungal cultures. This product was reinvestigated as an antibiotic in 1946, but its chemical structure was not finally resolved until 1957.[3] At present, the number of known antibiotics is large and continues to increase.

Secondary metabolites do not have only antibiotic activity; they can also be used against animals and against plants, as enzyme inhibitors, autoregulators, mineral scavengers and surfactants, and for light absorption.[4] Mycotoxins are typical secondary metabolites produced by filamentous fungi, and their biosynthesis involves the full range of secondary metabolic pathways, including polyketide, mevalonate and amino acid derived pathways, as well as combinations of the same.[5] They are produced by a wide range of fungi, including many which grow on raw food and food products. The reason for the secretion of these substances into the medium is not exactly understood; in fact, competition for the habitat and nutrients causes many species of fungi to excrete substances that inhibit growth and may even cause the death of organisms in their surrounding area, such as bacteria, fungi and insects. In a few cases these substances have a very specialised activity, being lethal for a particular group of life forms. When this occurs, they may be used to

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Fig. 1. Structure of aspyrone (**1**) and asperlactone (**2**).

control populations of certain species that are harmful to man, as is the case of agricultural antibiotics[6] and those used in human pharmacology.[7]

Microbial products are now being applied in every sphere of pesticide use. Thus, some antifungal, antibacterial, insecticidal and herbicidal products used in crop protection have been obtained from micro-organisms.[8]

Recent progress with microbial products as lead molecules for the development of new chemical classes of fungicidal compounds is very encouraging.[9] The appearance of many new and very diverse molecules with biological activity that are found primarily in filamentous fungi is a cause for optimism about the discovery of new molecules with new and interesting modes of action. The microbial world is more diverse and rich in compounds and activities than we can ever comprehend.[9]

The purpose of this study was to determine the activity of aspyrone (Fig. 1; **1**), a secondary metabolite of *Aspergillus ochraceus* Wilhelm, against some bacteria and fungi and to compare its inhibitory effect with the biological activity of asperlactone (**2**),[10,11] another *A. ochraceus* metabolite, and with some aspyrone derivatives (Fig. 2).[12]

2 MATERIAL AND METHODS

2.1 *Aspergillus ochraceus* strain and its metabolites aspyrone and asperlactone

The *A. ochraceus* strain, which is active against *Trichoderma sp.*, was isolated from an environmental contamination sample obtained in an apple-packing house in Lleida. This fungus was maintained at 4°C on PDA (potato dextrose agar) slants (composition per litre: infusion from 200 g of potatoes; glucose, 20 g; agar-agar, 15 g). The strain was shown to be an ochratoxin A producer according to the methods of Cvetnié & Pepeljnjak[13] and Ngiloriti & Kroll.[14]

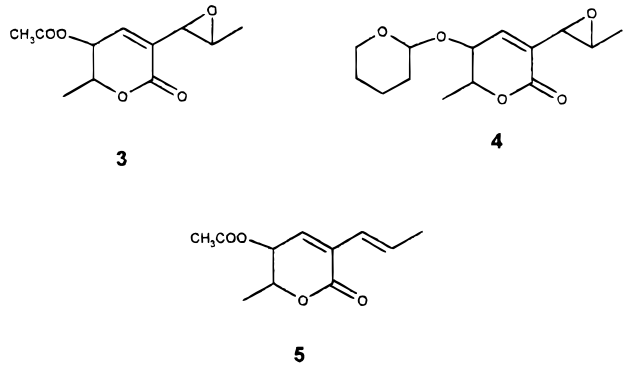

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Fig. 2. Structure of aspyrone derivatives (**3**, **4**, **5**).

A spore suspension was obtained by incubation of this mould in PDA for five to seven days at 28°C.

One millilitre of the suspension was inoculated into each of 50 Erlenmeyer flasks containing 100 ml of an aqueous solution of potassium dihydrogen phosphate, (1·0); magnesium sulfate heptahydrate, (0·5); potassium chloride (0·5); urea, (0·7) and dextrose (75 g litre^{-1}). The pH of the culture was adjusted to 5·5 before autoclaving. After 15 days of static incubation at 28°C, the mould broth was recovered by filtration and then extracted three times with ethyl acetate. The ethyl acetate solution was evaporated, and the residue was again dissolved in a few millilitres of diethyl ether. Then it was cooled to −20°C to crystallise out aspyrone (m.p. 102–104°C).

The mother liquor after aspyrone crystallisation was chromatographed on an open silica gel column (0·060–0·200 nm) using diethyl ether + hexane (7 + 3 by volume) as eluant. The product of the fractions immediately after those of aspyrone was collected and identified by TLC as asperlactone (TLC aluminium sheets aluminium oxide 60 F_{254} neutral (Type E) 20 × 20, Merck 5550; $R_f = 0{\cdot}5$ for aspyrone, $R_f = 0{\cdot}4$ for asperlactone, diethyl ether + hexane 9 + 1 by volume).[12]

2.2 Synthetic derivatives of aspyrone

Three synthetic derivatives of aspyrone were obtained:[12]

5-Acetoxy-3-(1,2-epoxypropyl)-6-methyl-5,6-dihydropyran-2-one (**3**)

3-(1,2-Epoxypropyl)-6-methyl-5-(tetrahydropyranyloxy)-5,6-dihydropyran-2-one (**4**)

5-Acetoxy-3-(1-propenyl)-6-methyl-5,6-dihydropyran-2-one (**5**)

2.3 Test micro-organisms

The following mycelial fungi were used: *Alternaria alternata* (Fr.) Keissler CECT 2662, *Aspergillus ochraceus* CECT 2948, *Botrytis cinerea* Pers. CECT 2100, *Fusarium oxysporum* Schlecht CECT 2159, *Rhizopus stolonifer* (Ehrenb.)Lind CECT 2344, and *Colletotrichum coccodes* (Wallr.) S. Hughes, *Helminthosporium monoceras* (Drechs.), *Penicillium expansum* Link., *P. mineoluteum* Dierck, *Phomopsis amygdali*, *Phytophthora citrophthora* (Sm.&Sm), *Trichoderma* sp1 and *Trichoderna* sp2 from our laboratory.

The yeast strains used in the study were: *Candida albicans* (Robin)Berkhout CECT 1394, *Kluyveromyces marxianus var marxianus* CECT 1123, and *Saccharomyces cerevisiae* Meyer ex Hansen CECT 1383.

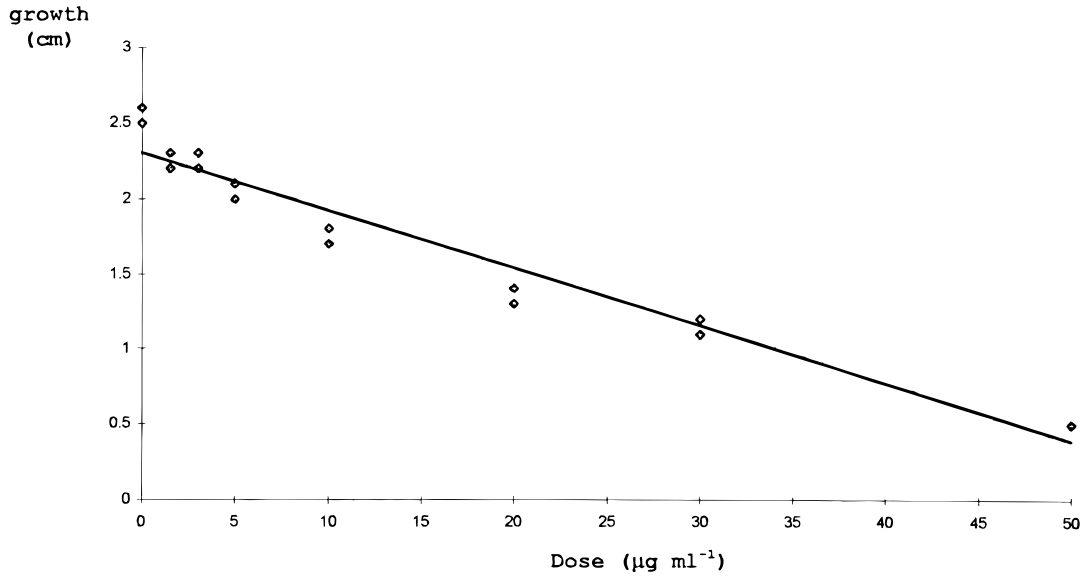

Fig. 3. Linear regression plot of radial growth of *Trichoderma* sp1 versus concentration of aspyrone in the growth medium ($Y = -0{\cdot}038X + 2{\cdot}304$; $R^2 = 0{\cdot}95$).

The following bacteria were used: *Arthrobacter simplex* Lochh. CECT 466, *Bacillus megaterium* deBary CECT 44, *Bacillus subtilis* (Ehrenb.) Cohn CECT 35, *Brevibacterium lipolitica* IAM 1398 (DSM 778), *Clavibacter michiganiensis* (Smith) Davis *et al.* CECT 79, *Erwinia amylovora* (Brussel) Winslow *et al.* CECT 222, *Enterobacter aerogenes* (Krzue) Hormaeche & Edwards CECT 684, *Enterobacter cloacae* (Jordan) Hormaeche & Edwards CECT 194, *Escherichia coli* (Mig.) Cast. & Chalm. CECT 405, *Mycobacterium phlei* Lehm. & Neum. CECT 3009, *Nocardia corynebacterium* CECT 409, *Pseudomonas aeruginosa* (Schroetr)Mig. CECT 326, *Rhodococcus rhodochrous* CECT 3012, *R. rhodochrous* CECT 3046, *Salmonella enteritidis* CECT 556, *Staphylococcus aureus* Rosenbach CECT 240 and *Bacillus subtilis* H17 and *Bacillus subtilis* M45 from Dr Yoshito Sadaure; Radioisotope Center, National Institute of Genetics, Mishima 411, Japan.

The meaning of the code numbers used for the species is as follows: CECT, Colección Española de Cultivos Tipo (Spanish Type Culture Collection), University of Valencia, Burjasot, Spain; IAM, Institute of Applied Microbiology, University of Tokyo, Japan; DSM, Deustche Sammlung von Microorganismen, Braunschweig, Germany.

2.4 Test for activity against mycelial fungi

The biological activity of compounds against mycelial fungi was assessed in radial growth tests on PDA medium. Each product was dissolved in 0·5 ml of acetone and included in 7·5 ml of the medium in 55-mm Petri dishes; for the control test 0·5 ml of the acetone was used. The assay was performed by placing a 4-mm diameter plug of growing mycelia onto the centre of a Petri plate containing the test material in the media. All treatments were replicated twice, and replicate plates were incubated at 28°C. The radial growth of mycelia in all plates was measured when the growth on the untreated plates neared the edge. Each data point is the mean of at least six measurements of a growing colony. The percentage growth inhibition was calculated from mean values. The activity of aspyrone against *Trichoderma* sp1 was performed at different concentrations: 0·0, 1·5, 3·0, 5, 10, 20, 30 and 50 μg ml^{-1}. The effective dose for inhibiting 50% of the mycelial growth was estimated by linear regression analysis. All other assays were done at concentrations of 20 μg ml^{-1}.

2.5 Test for activity against bacteria and yeasts

The paper-disc diffusion method was used. The cultures were prepared by inoculating a tube of nutrient broth with the micro-organisms to be tested, followed by incubation at 32°C for 18 h. Half a millilitre of the inoculum preparation was added to 15 ml of melted (46°C) Mueller-Hinton or yeast extract-peptone-glucose agar for bacteria or yeast respectively. The products were dissolved in acetone. Paper discs, 5 mm diameter, were impregnated with 10, 20, 50, 100 or 200 μg per disc. The discs were allowed to dry and then evenly spaced on the agar surface of each prepared plate. The plates were then inverted and preincubated at 4°C for 2 h to allow uniform diffusion into the agar. After preincubation, the plates were incubated for 24 h at 32°C. The lowest concentration necessary to form a zone of inhibition (μg per disc) was measured; the minimum width qualify as an inhibition zone was 9 mm.[15,16] The experiment involved the use of at least two plates for each test.

2.6 Statistical analysis

Fungal activities were subjected to analysis of variance utilizing the SAS 6.11 program. The Duncan's test was applied to determine the significance of differences between mean values when *F*-test rejected the hypothesis that the treatment means are all equal ($P \leqslant 0{\cdot}05$).

3 RESULTS AND DISCUSSION

Aspyrone was evaluated for activity against growth of *Trichoderma* sp1 at different concentrations. Figure 3 shows the dose-response curve of purified aspyrone; this was nearly linear and highly reproducible. It was found that, by extrapolation from linear regression analysis, aspyrone inhibited growth of *Trichoderma* by 50% at a concentration of 27 μg ml^{-1}. To test the biological activity against other moulds, a concentration of 20 μg ml^{-1} was chosen, which means a 47% growth reduction of *Trichoderma* sp1 (Table 1). At this level, only *Helminthosporium monoceras* was highly inhibited. Activity against *Botrytis cinerea*, *Colletotrichum coccodes*, *Fusarium oxysporum*, *Penicillium expansum*, *Phomopsis amygdali*, *Phytophthora citrophthora* and *Trichoderma* sp2 was similar, showing a 20–30% growth reduction, although Duncan's Test of the transformed means showed significant differences between some of these data. No significant reduction in the radial growth was found for *Alternaria alternata*, *Aspergillus ochraceus*, *Penicillium mineoluteum* or *Rhizopus stolonifer*.

To compare the inhibitory effect of aspyrone (**1**) with the biological activity of aspyrone derivatives (**3**, **4**, **5**), asperlactone (**2**) and captan, a known fungicide, five moulds were tested (Table 2). The aspyrone derivatives with a modification only in the hydroxyl group (**3**, **4**) retained activity against *Fusarium oxysporum* and *Colletotrichum coccodes*, but this disappeared when the epoxy group was removed (**5**). Asperlactone (**2**) also showed no activity against the two fungi. Captan was not active against *F. oxysporum* and showed only low activity against *C. coccodes*. Against *Helminthosporium monoceras*, aspyrone (**1**), its derivative obtained by tetrahydropyranilation of the alcohol group (**4**) and asperlactone (**2**) exhibited better results than derivatives **3** and **5**, and captan. Against *Trichoderma*, aspyrone (**1**) and compound **4** showed greater growth inhibition than the other substances assayed. With *Phytophthora citrophthora* the activity of derivative **4** was higher than the activity of aspyrone (**1**) and asperlactone (**2**) but lower than that of captan; no activity was found with derivative **5**, indicating that the epoxy group is important for this activity.

TABLE 1
Growth Reduction of Several Fungi on PDA at 20 μg ml^{-1} of Aspyrone (**1**)

Fungi	*Growth reduction* (%) ($\pm SE$)[a]
Trichoderma sp1	46·7 (±0·5)a
Alternaria alternata	0
Aspergillus ochraceus	0
Botrytis cinerea	26·7 (±0·5)b
Colletotrichum coccodes	30·9 (±0·4)c
Fusarium oxysporum	20·4 (±0·4)d
Helminthosporium monoceras	82·8 (±1·4)e
Penicillium expansum	27·9 (±0·4)b
Penicillium mineoluteum	0
Phomopsis amygdali	22·1 (±0·4)f
Phytophthora citrophthora	23·8 (±0·5)g
Rhizopus stolonifer	0
Trichoderma sp2	26·2 (±0·4)b

[a] Two replicates of each fungus with six measures per replicate were carried out. *S.E.* were calculated from original data, while analyses of variance were computed following arcsin $\sqrt{x}$ transformation. Means in the same column bearing a different letter are significantly different ($P \leq 0{\cdot}05$) according to Duncan's test.

TABLE 2
Growth Inhibition of Several Fungi by Aspyrone (**1**), Asperlactone (**2**), Some Aspyrone Derivatives (**3**, **4**, **5**) and Captan at a Concentration of 20 μg ml^{-1}

	Growth reduction (%) ($\pm SE$)[a]				
Compound	Trichoderma sp1	C. coccodes	F. oxysporum	H. monoceras	Ph. citrophtora
1	46·7 (±0·5)a	30·9 (±0·4)a	20·1 (±0·4)a	82·8 (±1·4)a	23·8 (±0·5)a
2	29·9 (±0·5)b	0	0	75·1 (±0·6)b	24·8 (±0·6)a
3	39·2 (±0·3)c	21·8 (±0·5)b	21·3 (±0·6)a	67·3 (±0·5)c	29·9 (±0·3)b
4	50·2 (±0·3)a	18·3 (±0·4)c	15·1 (±0·5)b	73·6 (±0·5)b	50·1 (±0·4)c
5	24·8 (±0·4)d	0	0	25·1 (±0·5)e	0
Captan	33·2 (±0·6)e	13·9 (±0·4)d	0	47·3 (±0·7)f	56·1 (±0·4)e

[a] Two replicates of each fungus with six measures per replicate were carried out. S.E. were calculated from original data, while analyses of variance were computed following arcsin $\sqrt{x}$ transformation. Means in the same column bearing a different letter are significantly different ($P \leq 0{\cdot}05$) according to Duncan's test.

TABLE 3
Minimal Inhibitory Dose of Aspyrone (**1**) against Some Bacteria and Yeasts

Micro-organisms	*Inhibition dose* (*μg per disc*)
Enterobacter aerogenes	100
Enterobacter cloacae	100
Escherichia coli	100
Erwinia amylovora	N.I.[a]
Salmonella enteritidis	50
Pseudomonas aeruginosa	200
Pseudomonas testosteroni	25
Bacillus megaterium	100
Bacillus subtilis	200
Bacillus subtilis H17	25
Bacillus subtilis M45	50
Staphylococcus aureus	100
Arthrobacter simplex	50
Brevibacterium lipolitica	200
Clavibacter michiganiensis	N.I.
Mycobacterium phlei	N.I.
Nocardia corynebacterium	N.I.
Rhodococcus rhodochrous 3012	200
Rhodococcus rhodochrous 3046	50
Candida albicans	100
Kluyveromyces marxianus	200
Saccharomyces cerevisiae	N.I.

[a] N.I., no inhibition at 200 μg per disc.

The antibiotic activity of aspyrone (**1**) was assayed against 19 bacteria and three yeasts (Table 3). Aspyrone showed a similar inhibition of all members of the *Enterobacteriaceae* group tested, except *Erwinia amylovora*, at a dose of 100 μg per disc or lower, as in *Salmonella enteritidis* (50 μg per disc) for example. A different bearing was shown by the two representatives of *Pseudomonas* investigated; *P. testosteroni* was inhibited at 25 μg per disc but *P. aeruginosa* required 200 μg per disc. Among Gram positive bacteria, *Bacillus subtilis* H17 was inhibited at the lowest assayed dose (25 μg per disc), followed by *Bacillus subtilis* M45, *Arthrobacter simplex* and *Rhodococcus rhodochrous*(CECT 3046) at quantities of 50 μg per disc. *Bacillus megaterium* and *Staphylococcus aureus* required a dose of 100 μg per disc; no inhibition was observed in *Mycobacterium phlei* or *Nocardia corynebacterium* when the disc was impregnated at 200 μg per disc.

The responses of the three assayed yeasts were very different; while *Kluyveromyces marxianus* was inhibited at a dose of 200 μg per disc and *Candida albicans* at 100 μg per disc, *Saccharomyces cerevisiae* did not show inhibition at the highest dose used.

To compare the antibiotic effect of aspyrone (**1**) with the biological activity of aspyrone derivatives, asperlactone (**2**) and terramicin, a known antibiotic, four bacteria and one yeast were tested. Table 4 shows that, in general, aspyrone (**1**) showed better activity than asperlactone (**2**) or aspyrone derivatives (**3**, **4**, **5**), although terramicin always showed the best activity against all bacteria. Some of these results, mainly the activity against *E. coli* and *S. aureus*, could be explained in terms of product solubility or by supposing the bioactive compound was not directly the lactone group but a product resulting from opening the ring. Again, the epoxy group seems to be necessary for the activity.

Several authors have worked on the synthesis of aspyrone derivatives, in general lactones. Some of them have shown bioactivity. Thus, podoblastines showed antifungal properties,[17] whereas fomolactone and asperline demonstrated antibiotic properties[18] and asmundolactone an antifeedant capacity against some insect larvae.[19] Without the lactone group but with a related aspyrone structure, R-avellanol showed antitumoral activity.[20] Aspyrone itself shows nematicidal activity towards *Pratylenchus penetrans* (Cobb),[21] whereas asperlactone was previously[12] described as a substance with a remarkable IGR activity against *Tribolium castaneum Hebst.* and activity against *Nezara viridula* L. Our results, as well as the nematicidal activity of aspirone (**1**) described recently, seem to indicate that aspyrone (**1**) could be used as a lead to find more active compounds. These compounds should maintain the epoxy group, introducing modifications to the position occupied by the hydroxy group or the primary carbon neighbour to the epoxide.

TABLE 4
Minimal Inhibitory Quantity of Aspyrone (**1**), Asperlactone (**2**), Aspyrone Derivatives (**3**, **4**, **5**) and Terramicin against Some Bacteria and Yeast

	Inhibition (*μg per disc*)					
Micro-organism	*1*	*2*	*3*	*4*	*5*	*Terramicin*
E. coli	100	100	200	N.I.	N.I.	10
P. aeruginosa	200	N.I.[a]	100	N.I.	N.I.	10
S. aureus	100	100	N.I.	200	N.I.	10
B. cereus	200	N.I.	200	N.I.	N.I.	10
C. albicans	100	N.I.	N.I.	N.I.	N.I.	N.I.

[a] N.I., no inhibition at 200 μg per disc.

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